

STAT1 and STAT3 mediate thrombin-induced expression of TIMP-1 in human glomerular mesangial cells

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STAT1 and STAT3 mediate thrombin-induced expression of TIMP-1 in human glomerular mesangial cells.

Background. Thrombin exhibits numerous biological effects on glomerular resident cells, such as cell proliferation, release and synthesis of cytokines and collagen, expressions of metalloproteinases and their inhibitors, especially tissue inhibitor of metalloproteinase-1 (TIMP-1). However, the signaling mechanisms underlying these cellular events have not been fully elucidated. The present study was designed to examine the role of signal transducers and activators of transcription (STAT) in thrombin-induced TIMP-1 expression in human mesangial cells.

Methods. Cultured human glomerular mesangial cells were incubated with thrombin up to 12 hours. The effects of the antisense of STAT1 and antisense of STAT3 on stimulated TIMP-1 mRNA levels and DNA-binding activities of both STAT1 and STAT3 were determined using Northern blot, electrophoretic mobility shift assay (EMSA), and supershift assay.

Results. Cultured human mesangial cells constitutively expressed TIMP-1, and thrombin induced TIMP-1 gene transcription in a time- and dose-dependent manner. Hirudin, a specific inhibitor of thrombin, could block thrombin-induced TIMP-1 expression. Thrombin also induced STAT-DNA binding activity in a similar time- and dose-dependent manner. In order to examine the role of STAT in thrombin-induced TIMP-1 expression, STAT1 and STAT3 antisense oligonucleotides were used. EMSA showed that STAT1 and STAT3 antisense oligonucleotides could inhibit both thrombin-induced STAT-DNA binding activities and TIMP-1 mRNA expression; the supershift assay showed that the SIF band consisted of STAT1 and STAT3 proteins.

Conclusions. Both STAT1 and STAT3 may be involved, at least in part, in thrombin-induced expression of the *TIMP-1* gene in cultured human mesangial cells.

Glomerular fibrin deposition is a documented feature of numerous renal diseases characterized by severe renal tissue injury [1]. Thus, excessive intraglomerular throm-

bin formation is expected in these conditions. Recent research has demonstrated that thrombin is a potent renal growth factor under renal pathophysiological conditions [2], and it might play an important role in the pathogenesis of proliferative glomerulonephritis. The role of thrombin in the progressive chronic renal diseases has not been fully understood. However, thrombin might well affect the pathophysiology of glomerulosclerosis, the common pathway of various kidney diseases, by influencing the enzymes for matrix degradation [3]. It has been reported that thrombin promotes the expression of tissue-type inhibitor of metalloproteinase-1 (TIMP-1), a potent inhibitor of the key matrix-degrading enzymes, matrix metalloproteinases, in cultured human mesangial cells [4].

While thrombin is well known to initiate multiple signaling pathways via its receptors [5], which signaling pathway is involved in thrombin-induced TIMP-1 expression has not been determined. Signal transducers and activators of transcription (STAT) is a recently characterized signaling pathway that regulates many cellular events induced by transforming growth factor- β (TGF- β), tumor necrosis factor- α (TNF- α), erythropoietin (EPO), interleukins (IL), etc. Thrombin stimulates STAT-DNA binding activity in rat aortic smooth muscle cells and human platelets, but inhibits STAT3 signaling induced by IL-6, leukemia inhibitory factor, and ciliary neurotrophic factor in CCL39 cells [6]. Since the binding site for STAT3 has been found in the promoter region of the *TIMP-1* gene [7], we hypothesized that thrombin-induced TIMP-1 expression is mediated through the STAT pathway, and tested this premise in the present study.

METHODS

Glomerular mesangial cell isolation and culture

Human glomerular mesangial cells were isolated from adult kidneys unsuitable for transplantation due to immunological reasons and cultured in RPMI 1640 (Gibco-BRL) supplemented with 20% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 μ g/mL strepto-

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mycin at 37°C in 5% CO₂. The cells were identified by their typical stellate morphology at subconfluence and typical hillocks at confluence, and were positively stained for actin, myosin, and type IV collagen, but negative for cytokeratin and factor VIII, by immunofluorescence [8]. Cells between passages 3 and 5 were used for the experiments. The cells at 80% confluence were deprived of serum for 12 hours, and then stimulated with thrombin in a serum-free RPMI 1640 medium for 6 to 12 hours. The final concentrations of thrombin were 0.5, 1.5, and 4.5 U/mL, respectively, and 9.0 U/mL of hirudin (Sigma), a specific inhibitor of thrombin was used when indicated. For time courses assays, cells were harvested after being incubated with thrombin (4.5 U/mL) for 0.5, 1, 3, 6, and 12 hours, respectively.

Northern blot analysis of thrombin-induced expression of TIMP-1 mRNA in glomerular mesangial cells

Total cell RNA was extracted with Trizol reagent (Gibco-BRL) according to the manufacturer's instruction. Briefly, cells were rinsed three times with cold phosphate-buffered saline (PBS) solution, 0.8 mL Trizol solution was added into the flask, and stood on ice for 15 minutes. The cell lysates were transferred into sterile centrifugation tubes, mixed with chloroform, and centrifuged at 12,000 rpm for 15 minutes at 4°C. The upper transparent layer was transferred to another centrifuge tube and mixed with equal volume of isopropanol, followed by centrifugation again at 4°C. The RNA pellets were dissolved in DEPC-treated water and quantified with spectrophotometer. Twenty micrograms of total RNA were electrophoresed on 1% agarose gel containing 2.2% formaldehyde and transferred onto nylon membrane (Hybond; Amersham) by capillary blotting, cross-linked in a ultraviolet cross-linker, pre-hybridized, and then hybridized with ³²P-labeled human TIMP-1 probe. The probe was prepared by reverse transcription-polymerase chain reaction (RT-PCR). The primers used for RT-PCR are as follows: human TIMP-1 sense sequence, 5'-TTG AAT TCC CAC CAT GGC CCC CTT TGA GCC-3'; antisense, 5'-GCA GGA TTC AGG CTA TCT GGG ACC GCA-3'. The PCR products were purified with glassmilk (Bio-Rad) and identified by DNA sequencing.

Antisense oligodeoxynucleotide studies

Phosphorothioated 21-mer oligodeoxynucleotides for STAT 1 and STAT3 were synthesized on an Applied Biosystem 394 synthesizer by using β-cyanoethylphosphoramidite chemistry to minimize degradation of the oligonucleotides by endogenous nucleases. The antisense oligonucleotides were directed against the translation start site (AUG codon) and surrounding nucleotides of the human *STAT1* gene and *STAT3* gene, respectively [9]. The STAT 1 antisense oligonucleotide sequence was

5'-CCA CTG AGA CAT CCT GCC ACC-3' and the corresponding sense oligonucleotide sequence was 5'-GGT GGC AGG ATG TCT CAG TGG-3'. The STAT 3 antisense oligonucleotide sequence was 5'-CCA TTG GGC CAT CCT GTT TCT-3' and the corresponding sense oligonucleotide sequence was 5'-AGA AAC AGG ATG GCC CAA TGG-3'. In addition, the scrambled oligonucleotide sequence was CAA ATG GGC TCC GAC GTC GGT.

To evaluate the role of STAT1 and STAT3 in thrombin-induced TIMP-1 expression, subconfluent mesangial cells were deprived of serum for 12 hours, followed by changing the medium to serum-free RPMI 1640 containing 3 μmol/L antisense oligonucleotides in 10 mg/mL lipofectin and incubated for 10 hours before adding thrombin. As controls, cells received serum-free RPMI 1640 medium containing lipofectin with/without 3 μmol/L sense, or scrambled oligonucleotides.

Preparation of nuclear proteins

The nuclear protein extracts were obtained according to the method of Dignam, Lebovitz and Roeder, with some modifications [10]. Briefly, cells were rinsed twice with cold PBS, scraped with a rubber policeman and transferred to microcentrifuge tubes. Cell pellets were suspended in 400 μL cold buffer A [10 mmol/L HEPES pH 7.9, 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol (DTT), 0.2 mmol/L phenylmethylsulfonyl fluoride (PMSF)] and left on ice for efficient cell swelling, followed by a centrifugation for 10 seconds. Nuclear pellets were re-suspended again in 40 μL cold buffer C [20 mmol/L HEPES pH 7.9, 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT, 0.2 mmol/L PMSF] and incubated on ice for 30 minutes. After centrifugation at 12,000 rpm for two minutes at 4°C, the concentrations of the supernatant proteins were determined. Aliquots of the nuclear proteins were stored at -70°C until use.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was carried out according to previous methods with modification [11]. The double-stranded STAT consensus oligonucleotides probe used in this study was m67 (5'-CATTTCCTGTAAATC-3'), which binds STAT1 and STAT3 [11], was end-labeled with γ-³²P-ATP by T4 polynucleotide kinase (Gibco-BRL) according to the manufacturer's protocol. For binding reaction, 50,000 cpm of the end-labeled STAT1 or STAT3 probe was co-incubated with a cocktail of 20 μg nuclear proteins in gel shift binding buffer (10 mmol/L Tris-HCl, pH 7.5, 50 mmol/L NaCl, 4% glycerol, 1.0 mmol/L MgCl₂, 5.0 mmol/L EDTA, 0.5 mmol/L DTT) and 2 μg poly (dI-dC) at room temperature for 30 minutes. EMSAs were performed on non-

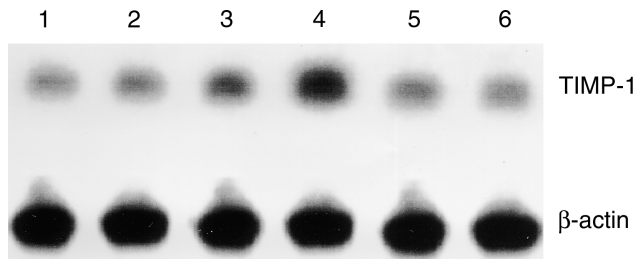


Fig. 1. Thrombin induced tissue inhibitor of metalloproteinase-1 (TIMP-1) in a dose-dependent manner. Cells were harvested after incubation with thrombin and/or hirudin for six hours. Lanes are: (1) control; (2–4) cells incubated with thrombin at 0.5, 1.5, and 4.5 U/mL, respectively; (5) cells treated with thrombin 4.5 U/mL plus hirudin 9 U/mL; (6) cells incubated with hirudin 9 U/mL only.

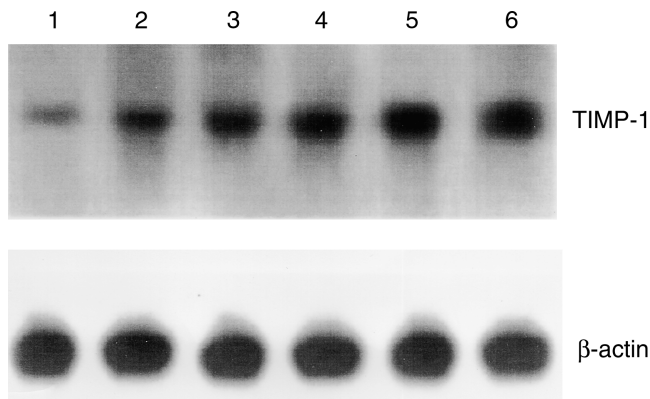


Fig. 2. Thrombin induced TIMP-1 mRNA expression in a time-dependent manner. Cells were harvested after incubation with 4.5 U/mL thrombin for 0 hour (lane 1), 0.5 hours (lane 2), 1 hour (lane 3), 3 hours (lane 4), 6 hours (lane 5), and 12 hours (lane 6).

denaturing 4% polyacrylamide gel in $0.5 \times$ TBE buffer at 100 V. Dried gels were subjected to autoradiography at -70°C . As controls, unlabeled oligonucleotides at 50-fold molar excess were included. In addition, three oligonucleotides incorporating distinct mutations, insertions, or deletions in the above STAT consensus oligonucleotides were also end-labeled to be used as radiolabeled probes. These mutant STAT probes, with mutated nucleotide(s) in *italics*, were: MUT1, 5'-CATTTCCCGTAAATC-3'; MUT2, 5'-CATTTCCATGCATGCATCGTAAATC-3'; MUT3, 5'-CATTT(X)AAATC-3'. Each mutant incorporated a distinct sort of change. MUT1 has a one-nucleotide mutation in the 5' side of the m67 probe; MUT2 has a 10-bp insertion separating the 5' and 3' sides of m67 probe; MUT3 deletes 5-bp between the 5' and 3' sides of m67 probe. For supershift assays, nuclear extracts were preincubated with STAT1 α & β polyclonal antibody (sc-346; Santa Cruz) and/or STAT3 polyclonal antibody (sc-482; Santa Cruz) for 30 minutes on ice after the addition of the probe.

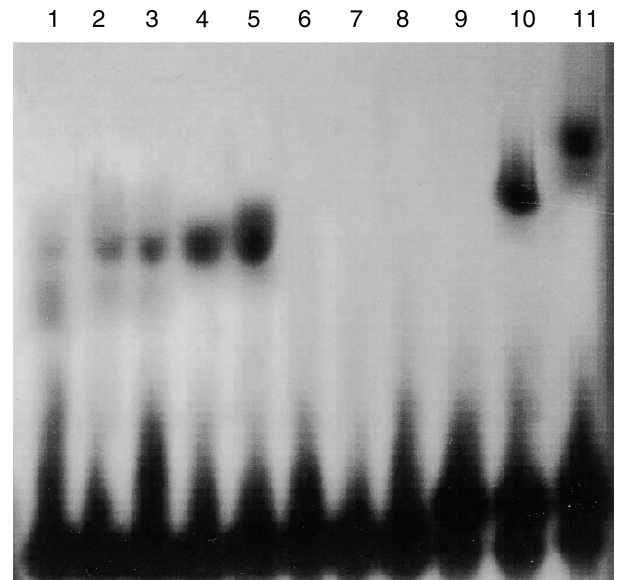


Fig. 3. Thrombin induced STAT-DNA binding in a dose-dependent manner. Cells were harvested after incubation with thrombin for six hours. Lanes are: (1) control; (2–5) cells incubated with thrombin at 0.5, 1.5, 3.0 and 4.5 U/mL, respectively. In lane 6, a 50-fold molar excess of unlabeled competitor probe was added as a cold competitor. Lanes 7–9, the radiolabeled m67 probe mutants 1, 2, and 3 did not form complexes with nuclear extracts from 4.5 U/mL thrombin-treated cells. Polyclonal anti-STAT1 (lane 10) or anti-STAT3 (lane 11) antibodies were added to nuclear extracts from cells pretreated with 4.5 U/mL thrombin prior to the addition of the probes. The sis-inducible factor (SIF) complex and the supershifted complex are indicated by the asterisk.

RESULTS

Thrombin induced TIMP-1 mRNA expression

Human glomerular mesangial cells constitutively expressed TIMP-1 mRNA. Thrombin up-regulated TIMP-1 gene expression in both dose- and time-dependent manners, and this could be inhibited by hirudin, a specific inhibitor of thrombin. Levels of TIMP-1 mRNA expressed by cells treated with 0.5, 1.5, and 4.5 U/mL thrombin were 1.5-, 2.8-, and 4.4-fold those of the control group. Cells treated with 4.5 U/mL thrombin together with 9.0 U/mL hirudin induced a less pronounced increase of TIMP-1 mRNA compared with that in the control (Fig. 1). The expression of TIMP-1 expression peaked when the cells had been incubated for six hours with 4.5 U/mL thrombin (Fig. 2).

Thrombin stimulated STAT-DNA binding activity

To explore the possible participation of STATs in thrombin-induced TIMP-1 gene expression, EMSA was performed to investigate the activity of STAT in human mesangial cells treated with or without thrombin. The results showed that thrombin promoted STAT-DNA binding activity in both dose- and time- dependent (Figs. 3 and 4). The outcome of applying cold or mutant com-

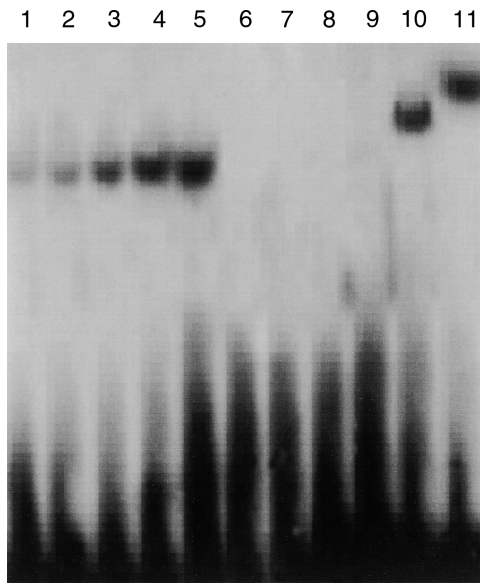


Fig. 4. Thrombin-induced STAT-DNA binding in a time-dependent manner. Cells were harvested after being incubated with 4.5 U/mL thrombin for 0 hour (lane 1), 0.5 hours (lane 2), 1 hour (lane 3), 3 hours (lane 4), 6 hours (lane 5). In lane 6, a 50-fold molar excess of unlabeled competitor probe was added as a cold competitor. Lanes 7–9, the radiolabeled m67 probe mutants 1, 2, and 3 did not form complexes with nuclear extracts from 4.5 U/mL thrombin-treated cells. Polyclonal anti-STAT1 (lane 10) or anti-STAT3 (lane 11) antibodies were added to nuclear extracts from cells pretreated with 4.5 U/mL thrombin prior to the addition of the probes. The sis-inducible factor (SIF) complex and the supershifted complex are indicated by the asterisk.

petitor probes supported the specificity of the m67 probe used. Supershift results demonstrated that the SIF band consisted of STAT1 and STAT3. In order to determine whether STAT1 and STAT3 are involved in thrombin-induced STAT-DNA binding activity, antisense oligonucleotides for STAT1 or STAT3 were added in the media to suppress the corresponding activities, respectively. EMSA showed that both STAT1 and STAT3 antisense oligonucleotides significantly diminished the STAT-DNA binding induced by thrombin, while the STAT1 or STAT3 sense or scrambled oligonucleotides had no such suppressing effects on thrombin-induced STAT-DNA binding activity. The supershifted bands were STAT1 or STAT3 as indicated. (Figs. 5 and 6).

Role of STAT 1 and STAT 3 in thrombin-induced TIMP-1 gene expression

To further identify the relationship between STAT activity and TIMP-1 gene expression, blocking experiments were used in this study. The role of thrombin in increasing TIMP-1 mRNA levels in human glomerular mesangial cells was inhibited by pre-incubation of the cells with STAT 1 or STAT 3 antisense oligonucleotides. STAT1 or STAT3 sense or scrambled oligonucleotides had no effects on TIMP-1 mRNA expressions. (Fig. 7).

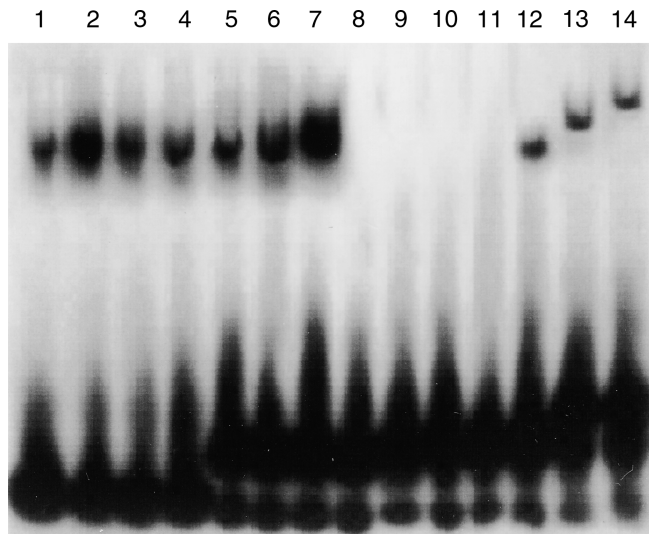


Fig. 5. STAT1 antisense inhibited STAT-DNA binding activity in glomerular mesangial cells. Subconfluent mesangial cells were deprived of serum for 12 hours, and the medium was changed to serum-free RPMI 1640, which contained 3 μ mol/L antisense oligonucleotides in 10 mg/mL lipofectin and incubated for 10 hours before adding thrombin. As controls, cells received serum-free RPMI 1640 medium containing lipofectin with or without 3 μ mol/L sense, or scrambled oligonucleotides. Cells were harvested after incubation with thrombin and/or hirudin for 6 hours. Lanes are: (1) control; (2) thrombin 4.5 U/mL; (3) thrombin 4.5 U/mL plus hirudin 9 U/mL; (4) antisense 3 μ mol/L; (5) sense 3 μ mol/L; (6) thrombin 4.5 U/mL plus antisense 3 μ mol/L; (7) thrombin 4.5 U/mL plus sense 3 μ mol/L; (8) 50-fold molar excess of unlabeled competitor probe as cold probes; (lanes 9 to 11) radiolabeled mutant probes 1, 2 and 3; (12) scrambled oligonucleotides 3 μ mol/L; (13) anti-STAT1 antibody; (14) anti-STAT3 antibody.

DISCUSSION

Many glomerular diseases are characterized by accumulation of extracellular matrix. Imbalance between its synthesis and degradation might contribute to the development of glomerulosclerosis. Glomerular resident cells have the ability to synthesize and secrete both matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) including TIMP-1, -2, -3 and -4. Abnormal expression of these enzymes and/or their inhibitors may induce destruction or remodeling of glomerular architecture under pathophysiological conditions [12–14].

TIMP-1 expression can be up-regulated in fibroblasts by a variety of soluble factors including interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α), epidermal growth factor (EGF), transforming growth factor- β (TGF- β) phorbol esters, and retinoic acid [15]. Recently, it has been reported that thrombin can increase the expression of TIMP-1 in cultured human mesangial cells [4]. It is well known that thrombin initiates multiple signaling pathways via its receptors [5]. However, it is still unknown which signaling pathway is involved in thrombin-induced TIMP-1 expression. Signal transducers and activators of transcription (STAT) is a recently

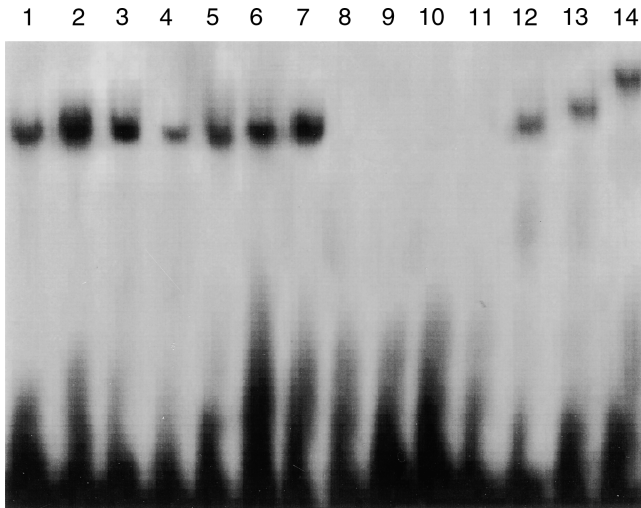


Fig. 6. STAT3 antisense also inhibited STAT-DNA binding activity in glomerular mesangial cells. Cells were incubated in the serum-free RPMI 1640 containing 3 $\mu\text{mol/L}$ antisense oligonucleotides in 10 mg/mL lipofectin and incubated for 10 hours before adding thrombin 4.5 U/mL. As controls, cells received serum-free RPMI 1640 medium containing lipofectin with/without 3 $\mu\text{mol/L}$ sense, or scrambled oligonucleotides. Cells were harvested after having been incubated with thrombin and/or hirudin for six hours. Lanes are: (1) control; (2) thrombin 4.5 U/mL; (3) thrombin 4.5 U/mL plus hirudin 9 U/mL; (4) antisense 3 $\mu\text{mol/L}$; (5) sense 3 $\mu\text{mol/L}$; (6) thrombin 4.5 U/mL plus antisense 3 $\mu\text{mol/L}$; (7) thrombin 4.5 U/mL plus sense 3 $\mu\text{mol/L}$; (8) 50-fold molar excess of unlabeled competitor probe as cold probes; (lanes 9 to 11) radiolabeled mutant probes 1, 2 and 3; (12) scrambled oligonucleotides 3 $\mu\text{mol/L}$; (13) anti-STAT1 antibody; (14) anti-STAT3 antibody.

characterized signaling pathway that regulates many cellular events induced by TGF- β , TNF- α , erythropoietin, interleukins, and others. Activation of STAT proteins has been reported in cells treated with various cytokines, growth factors, insulin, and angiotensin II [16]. Thrombin stimulated STAT-DNA binding activity in rat aortic smooth muscle cells and human platelets, but inhibited STAT3 signaling induced by IL-6, leukemia inhibitory factor, and ciliary neurotrophic factor in CCL39 cells [6]. It has been reported that there is a binding site for STAT3 in the promoter region of the *TIMP-1* gene [7]. Therefore, the present study was designed to test if thrombin-induced TIMP-1 expression might be mediated through STAT pathway.

The present study found that thrombin up-regulated TIMP-1 gene expression in cultured human mesangial cells. This effect was in both dose- and time-dependent manners and could be blocked by hirudin, a thrombin specific inhibitor. The latter proved the specificity of thrombin-induced TIMP-1 expression. These results are comparable to the previous report by Kaizuka et al [4]. In addition, the results showed that thrombin could promote STAT-DNA binding activity in both dose- and time-dependent manners in human mesangial cells. These data were consistent with Bhat et al's findings that

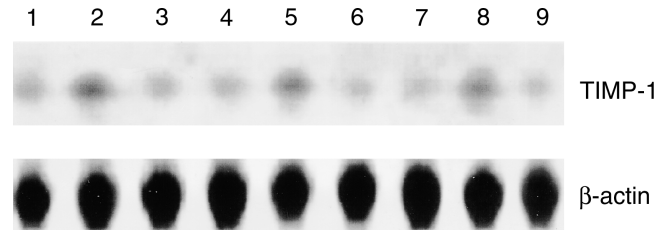


Fig. 7. STAT1 and STAT3 antisense oligonucleotides inhibited thrombin-induced TIMP-1 mRNA expression in glomerular mesangial cells. Cells were incubated in the serum-free RPMI 1640 containing 3 $\mu\text{mol/L}$ antisense oligonucleotides in 10 mg/mL lipofectin and incubated for 10 hours before adding thrombin 4.5 U/mL. As controls, cells received serum-free RPMI 1640 medium containing lipofectin with/without 3 $\mu\text{mol/L}$ sense, or scrambled oligonucleotides. Cells were harvested after having been incubated with thrombin and/or hirudin for six hours. Lanes are: (1) control; (2) thrombin 4.5 U/mL; (3) STAT3 antisense 3 $\mu\text{mol/L}$; (4) STAT3 antisense 3 $\mu\text{mol/L}$ plus thrombin 4.5 U/mL; (5) STAT3 sense 3 $\mu\text{mol/L}$ plus thrombin 4.5 U/mL; (6) STAT1 antisense 3 $\mu\text{mol/L}$; (7) STAT1 antisense 3 $\mu\text{mol/L}$ plus thrombin 4.5 U/mL; (8) STAT1 sense 3 $\mu\text{mol/L}$ plus thrombin 4.5 U/mL; (9) scrambled oligonucleotide 3 $\mu\text{mol/L}$.

thrombin promoted STAT activity in SMC, but were in contrast to Bhat et al's findings in CCL39 cells and lung fibroblasts [6, 17, 18]. The results of inhibition assay with antisense oligonucleotides of STAT1 or STAT3, in combination with the results of the supershift assay with polyclonal antibodies to STAT1 or STAT3, demonstrated that the major DNA-binding components were heterodimer STAT3/STAT1. In order to identify the relationship between STAT activation and TIMP-1 gene expression induced by thrombin, Northern blot analysis was carried out to examine TIMP-1 mRNA levels in different transfected groups in the presence of thrombin. Our data showed that pre-incubation with STAT1 or STAT3 antisense oligonucleotides also prevented thrombin induced TIMP-1 mRNA expression.

In summary, the present study suggests that STAT1 and STAT3 can be activated by thrombin and, at least in part, be involved in thrombin-induced TIMP-1 mRNA expression in human mesangial cells. This may be helpful in further understanding the pathophysiological behavior of thrombin in glomerular injury.

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